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Expression and mutagenesis of recombinant cholera toxin A subunit

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ADP-ribosylating protein exotoxins from *Vibrio cholerae* (CT) and *Escherichia coli* (LT-I) share two short regions of sequence similarity with *Bordetella pertussis* toxin (PT). Previous studies have indicated that substitution of arginine for lysine 7 within the first region of CT drastically decreases ADP ribosyltransferase activity. We have more closely defined the role of other amino acids in this region by generating modified proteins in which arginine 7 was replaced with lysine (R7K), aspartate 9 was replaced with arginine (D9R), glycine was substituted for proline 12 (P12G), amino acids 6 to 13 were deleted (Δ613) or the C-terminal KDEL sequence was changed to NEDL. The modified proteins R7K, D9R and Δ613 exhibited undetectable ADP ribosyltransferase activity. Comparison of the tryptic digest of R7K with native CT suggested that changes in protein conformation may be responsible for the loss of ADP-ribosylation activity.

Key words: Cholera toxin; PCR mutagenesis; ADP-ribosylation; KDEL; recombinant CT-A.

Introduction

Vibrio cholerae is the etiologic agent of cholera, an endemic disease of the Middle East which has recently become epidemic in Latin America.[1] The profuse, watery diarrhea, which is the main symptom of cholera, is the result of irreversible intoxication of the epithelial cells of the small intestine with an extracellular protein exotoxin, the cholera toxin (CT). CT holotoxin is an 84 kDa protein composed of five identical B chains and a 27 kDa A subunit (CT-A) which, in *V. cholerae*, is proteolytically nicked to form the enzymatically active A1 (22 kDa) and A2 (5 kDa) chains linked by a disulfide bond.[2]

The enzymatically active A subunits of CT and *E. coli* heat labile toxin (LT-I) share nearly 80% predicted amino acid sequence, a similar mode of action and the GM_1 cellular receptor.[3] CT-A and LT-I share only two short regions of similarity with the

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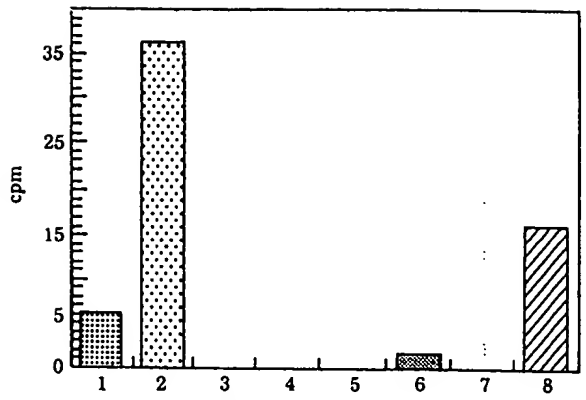


Fig. 1. Results of representative assays of ADP-ribosyltransferase activity of periplasmic fractions of *E. coli* BL21. Lane 1, 2 μg purified CT-A; lane 2, BL21:pNPCT; lane 3, BL21:pYS3; lane 4, BL21:R7K; lane 5, BL21:D9R; lane 6, BL21:P12G; lane 7, BL21:Δ613; lane 8, BL21:NEDL. Lanes 2–8 each contained 50 μg protein. Background counts were subtracted from the data. CPM are in thousands.

pertussis toxin (PT) of *Bordetella pertussis*. These two eight amino-acid regions, which span residues 6 to 13 and 60 to 67 in CT and LT-I, are located within the N-terminal region of the enzymatically active subunits of CT, LT-I and PT.[4] Site-specific mutagenesis of specific residues within the first region in all three toxins indicates that some amino acids appear to be critical for expression of full biological activity.[5–12] In particular, substitution of the arginine residue at position 9 in PT and position 7 in LT-I and CT reduced enzymatic activity of the recombinant A subunits below detectable levels.[6–9]

Our purpose in this study was to define more closely the role of other amino acids within the first region of sequence similarity and at the C-terminal end of the toxin.

Results

Assays of ADP-ribosylation activity

Biological activity of recombinant CT-A proteins detected in the periplasmic fractions is shown in Fig. 1. Of the proteins which had point mutations inside the first region of homology (Table 1), only BL21:P12G consistently demonstrated enzymatic activity above background. No detectable activity was seen in assays of the modified proteins BL21:R7K, BL21:D9R or BL21:Δ613.

Table 1 Amino acid sequence of the first region of sequence similarity and specific amino acid alterations made within it

	Tyr6 Arg7 Ala8 Asp9 Ser10 Arg11 Pro12 Pro13
R7K[a]	----------Lys----------
D9R	----------Arg----------
P12G	----------Gly----------
Δ613	Deletion of amino acids 6 to 13
NEDL	Substituted for KDEL in C-terminus

Δ613 deletion still resulted in translational read-through.
[a] Designations for mutant protein.

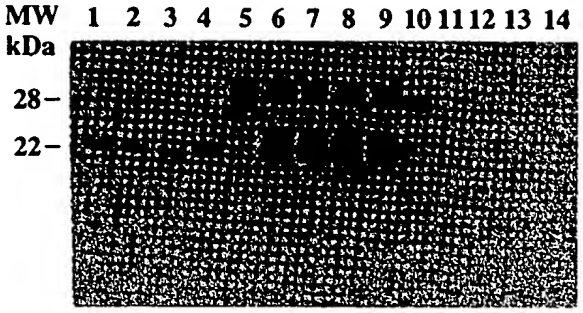


Fig. 2. Immunoblot of a limited tryptic digestion of CT-A from *V. cholerae* (lanes 1–4), BL21:pNPCT (lanes 5–9), and BL21:R7K (lanes 10–14). Proteins were treated with trypsin for 0, 15, 30 or 60 min before PMSF was added. Samples of unmodified recombinant CT-A and the R7K protein untreated with trypsin are also shown (lanes 5 and 10, respectively). Anti-CT-A polyclonal rabbit antisera was used to detect protein.

Limited tryptic digestion

To determine whether the dramatic decline in biological activity seen in the BL21:R7K protein might be due to conformational alterations in the mature protein, a limited trypsin digestion of the proteins was performed (Fig. 2). Because naturally occurring CT-A is already cleaved into A1 and A2 in *V. cholerae*, trypsinolysis of purified CT-A produced only the single 22 kDa protein band representing the A1 subunit even after 60 min of digestion (Fig. 2, lanes 1–4). The 5 kDa A2 subunit was too small to appear on the gel. Unmodified recombinant CT-A was rapidly cleaved to generate a protein which co-migrated with purified CT-A (A1) (Fig. 2, lanes 6–9). Digestion of the R7K protein with trypsin resulted in the formation of a number of smaller immunoreactive proteins (Fig. 2, lanes 11–14).

Expression of recombinant CT-A

The relative levels of expression of immunoreactive, recombinant CT-A proteins in the periplasmic fractions are shown in Fig. 3(a). Recombinant proteins demonstrated a slower electrophoretic mobility compared to the naturally occurring purified CT-A, suggesting that they were not cleaved into the A1–A2 conformation found in *V. cholerae*.

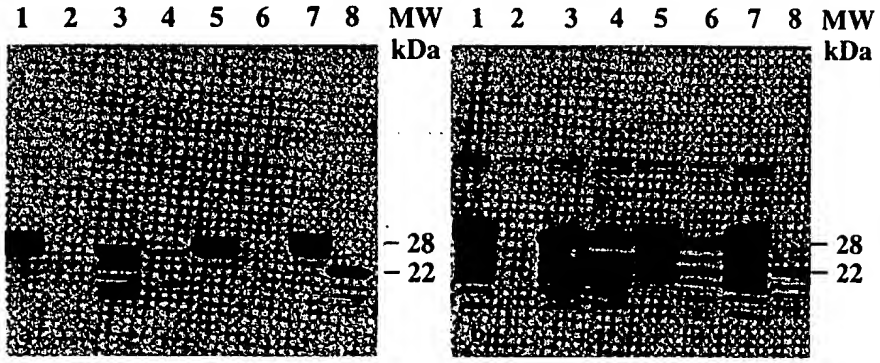


Fig. 3. Immunoblot of periplasmic (a) and cytoplasmic (b) recombinant CT-A proteins. Equivalent amounts of protein were boiled for 5 min in loading buffer containing β-mercaptoethanol, then electrophoresed on 4–20% gradient SDS–polyacrylamide gels (Schleicher and Schuell, Keene, NH) and blotted by standard methods.[22] Lane 1, BL21:pNPCT; lane 2, BL21:pYS3; lane 3, BL21:R7K; lane 4, BL21:D9R; lane 5, BL21:P12G; lane 6, BL21:Δ613; lane 7, BL21:NEDL; lane 8, purified CT-A. Anti-CT-A polyclonal rabbit antisera was used to detect protein.

Immunoreactive proteins produced by BL21:pNPCT (lane 1), BL21:P12G (lane 5) and BL21:NEDL (lane 7) appear primarily as single bands of approximately 27 kDa, the expected molecular mass of unnicked CT-A. BL21:R7K (lane 3) and BL21:D9R (lane 4) appear to be present in smaller quantities in the periplasmic fractions than other recombinant CT-A proteins, and the R7K protein in particular consistently demonstrated several smaller immunoreactive protein bands. Purified CT-A (lane 8) represents the nicked (A1) form of the toxin, with a molecular mass of approximately 22 kDa. Neither BL21:pYS3 (lane 2), which contained no CT sequences and served as a negative control, nor BL21:Δ613 (lane 6), lacking the eight amino acids of the first region of protein sequence similarity, demonstrated any immunoreactive protein in the periplasmic fractions. But comparison of immunoblots of the periplasmic and cytoplasmic fractions [Fig. 3(a), (b)] clearly indicates that all of the BL21 extracts containing *ctxA* are capable of producing immunoreactive CT-A protein, including BL21:Δ613, which was not apparent in Fig. 3(a). The level of expression varies considerably, however, as BL21:R7K, BL21:D9R, BL21:D613 appear to produce less immunoreactive toxin than BL21:pNPCT (lane 1), BL21:P12G (lane 5) and BL21:NEDL (lane 7). The higher molecular mass bands, which appear only in the cytoplasmic fractions, are probably *E. coli* proteins that cross-react with the polyclonal rabbit antisera but are unrelated to the CT product, since they are also visible on immunoblots of BL21 cytosolic preparations without the plasmid (data not shown).

Discussion

Using PCR mutagenesis techniques, we substituted lysine for arginine at position 7 (R7K) and arginine for aspartic acid at position 9 (D9R). The proteins resulting from the R7K and D9R substitutions had no detectable ADP-ribosylation activity, while alteration of proline to glycine at position 12 (P12G) did not eliminate enzymatic activity of the protein (Fig. 1). Deletion of the entire first region of protein similarity (Δ613) resulted in a protein that had no detectable enzymatic activity and was not detectable by Western blot in the periplasmic compartment (Figs 1 and 3). To confirm the lack of enzymatic activity of BL21:Δ613, we performed ADP-ribosyltransferase assays on cytoplasmic extractions of all recombinant proteins. A similar pattern of biological activity was seen in cytoplasmic and periplasmic samples, with BL21:Δ613 exhibiting no activity above background (data not shown). Alteration of the C-terminal KDEL protein sequence to NEDL did not appear to have any effect on enzymatic activity or on protein export (Figs 1 and 3). Proteolytic digestion of BL21:R7K confirmed studies performed with PT and LT-I[9] which showed that the substitution of lysine for arginine increased the susceptibility of the toxin to tryptic digestion, suggesting that the R7K substitution may produce an alteration of the tertiary structure of the mature toxin (Fig. 2).

The two modified proteins that demonstrated the greatest loss of biological activity (BL21:R7K and BL21:D9R) represent the most conservative substitutions in CT-A. The arginine at position 7, which was demonstrated in previous papers to be a critical site for ADP-ribosylation activity of PT, CT and LT-I, was replaced by lysine; these amino acids have identical charges and similar side-chain lengths. The loss in activity demonstrated in these studies is consistent with the decreased activity of the same alterations in PT,[6,8] in LT-I[9] and in CT.[7] Therefore, the conserved arginine residue within the eight amino acid region of sequence similarity appears to play a critical role in enzymatic activity of these ADP-ribosylating toxins.

Substitution of arginine for aspartic acid 9 (D9R) represents an increase in the side-chain length of the amino acid as well as an increase in the charge of the R group at

neutral pH. Barbieri and Cortina[5] substituted a serine residue for Asp at this position in the PT S1 molecule and were similarly unable to detect any ADP-ribosyltransferase activity.

The P12G substitution of glycine for proline at position 12 represents a potentially much more significant alteration in protein conformation. The ring structure of the imino acid proline drastically limits rotation about the N-C^{α} peptide bond, inducing torsional strain on the molecule. Proline residues are often associated with reverse turns or, when adjacent as in this case, may be part of a poly(Pro) helix. In any case, such a drastic structural alteration as Pro to Gly might be expected to produce a significant alteration in protein structure. This could affect the biological activity of the toxin if the tertiary structure of the protein and not simply the primary sequence is important for enzymatic activity. The BL21:P12G protein consistently demonstrated ADP-ribosyltransferase activity comparable to that of the unmodified CT-A; therefore the Pro-Pro dipeptide is probably not indicative of a poly(Pro) helical structure, which can involve as few as two proline residues and which would very likely be disrupted by the substitution of glycine. Proline and glycine residues are often found associated with reverse turns; it is possible that substitution of glycine for a proline residue at this position would have no effect on a reverse turn in CT-A and therefore no major decrease in biological activity would be seen.[13]

The deletion of all eight amino acids representing the entire first region of sequence similarity resulted in retention of the BL21:Δ613 protein in the cytoplasm [Fig. 3(a), (b)]. Assays of cytosolic fractions of the recombinant protein demonstrated that this protein has no detectable activity above background levels. Studies on PT by Cieplak *et al.*[14] and Barbieri and Cortina[5] also indicated that these residues are of critical importance for enzymatic activity. Since this protein does not appear to be exported from the cytoplasm, it is possible that removal of so many residues near the N-terminus of the protein may remove topogenic sequences essential for proper export as well as deleting residues required for enzymatic activity, regardless of the effect on the tertiary structure of the toxin.

The final four amino acids of the CT-A protein are KDEL, a protein motif which has been identified as a retention signal for soluble proteins in the endoplasmic reticulum of a wide variety of eukaryotic cells.[15] In the *Pseudomonas* exotoxin, which also has ADP-ribosylation activity, C-terminal KDEL residues appear to be critical for cytotoxic, but not ADP-ribosyltransferase, activity and are also involved in translocation of the exotoxin.[16] It has been suggested that the sequences KDEL of CT-A and RDEL of LT-I may be recognition sequences involved in translocation of the toxins into the cytosol and possibly targeting of toxin molecules within eukaryotic cells.[17] This may help to explain how CT-A, entering ileal cells at the luminal surface, is able to interact with the adenyl cyclase complex at the basolateral surface. Our C-terminal modified protein, in which the KDEL sequence was changed to NEDL, appeared to be exported at least as efficiently as the unmodified recombinant protein as judged by immunoblots of cytoplasmic and periplasmic fractions [Fig. 3(a), (b)]. This protein also exhibited ADP-ribosyltransferase activity at levels nearly equivalent to that of the unmodified CT-A, suggesting that in CT-A as in *Pseudomonas* the KDEL sequence is not involved in ADP-ribosylation activity.

When the two, small, highly conserved regions of protein sequence similarity near the N-terminus of the enzymatically active subunits of PT, CT and LT-I were first identified, it seemed likely that such sequence similarity in toxins which ADP-ribosylate regulatory proteins of the adenyl cyclase complex might be important for enzymatic activity. Site-specific mutagenesis studies performed by Barbieri and Cortina,[5] Lobet *et al.*[8] and Cieplak *et al.* appear to confirm the significance of the first

region of similarity and specific residues therein. Subsequent mutagenesis in the PT, CT-A and LT-I molecules has demonstrated, however, that point mutations in other parts of these proteins can have dramatic effects on toxicity,[10,11,18] either because the residues are actually involved in some phase of the catalytic activity of the toxin or because their alteration induces conformational changes in the protein, which inhibit enzymatic activity or holotoxin formation. Limited tryptic digestion of the unmodified CT-A and the R7K proteins demonstrated that the substitution of lysine for arginine at position 7 in CT-A increases the number of trypsin sites available in the altered construct. This is in contrast to results reported by Burnette *et al.*,[7] who were unable to detect any differences in proteolytic degradation in recombinant CT-A_1 of the R7K modified protein, but confirms the findings of Lobet *et al.*[9] with LT-I.

Materials and methods

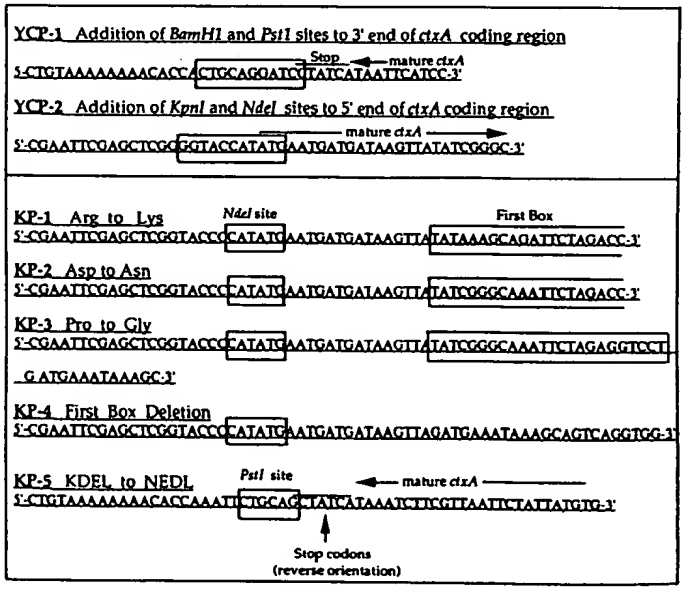
Materials. Restriction endonucleases and other DNA-modifying enzymes were purchased from GIBCO-BRL, Boehringer Mannheim or New England Biolabs and were used according to manufacturers' instructions.

E. coli strains. Transformation-competent DH5α bacterial cells (GIBCO-BRL) were used as initial transformants for all recombinant plasmids. BL21 bacteria[19] were used for expression of recombinant proteins.

Construction of CT-A expression vectors. *ctxA* was subcloned from pRT41[2] (kindly provided by Dr. John Mekalanos, Harvard University, Boston, MA) and expressed in a previously described plasmid, pYS3.[20]

Unique *Nde*I and *Pst*I sites were created in the CT-A gene using polymerase chain reaction (PCR) mutagenesis as described by Higuchi[21] using oligonucleotide primers listed in Table 2.

Table 2 YCP-1 and YCP-2 oligonucleotide primers were used to add *Pst*I or *Nde*I sites to the 3′ and 5′ terminii of the *ctx*A gene, respectively, by PCR mutagenesis of pNPCT



Primers KP1-5 were used to generate specific mutations in *ctxA* by pairing YCP-2 with Kp-1, -2, -3 and -4, and YCP-1 with KP-5.

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The *ctxA* gene was ligated into *Nde*I-*Pst*I sites of pYS3 to produce the plasmid pNPCT. The *ctxA* sequences were ligated into this plasmid in such a way as to remove the native *ctx* leader sequence, so that none of the resulting altered *ctx* genes were preceded by any leader sequence. Specific mutations were created as described in the legend to Table 2. Duplex DNA was amplified, digested with *Nde*I and *Pst*I and ligated into pYS3. The nucleotide sequence of each altered *ctxA* construct was verified by dideoxy sequencing using standard methods.[22]

Antibodies. CP7-3003F7 (3F7) is an IgG3 monoclonal antibody specific for a synthetic peptide representing amino acids 6 to 17 of PT (Dr. James Kenimer, Center for Biologics, Food and Drug Administration, Bethesda, MD). Polyclonal rabbit antisera generated against CT-A (generously provided by Dr. Joel Moss, NIH, Bethesda, MD) was used to detect expression of modified recombinant CT-A proteins by standard methods.[22]

CT-A induction. Plasmids containing the *ctxA* constructs were introduced into transformation competent BL-21 bacteria. Colonies carrying the recombinant plasmids were grown in Luria Broth with 100 μg/ml ampicillin at 37°C with shaking to $OD_{600} = 0.8–1.0$. IPTG (1 mM final concentration) was added and shaking was continued for a further 120 min. Cells were harvested by centrifugation at $4000 \times g$ for 10 min at 4°C and suspended in 20% sucrose, incubated on ice for 10 min, centrifuged as before, suspended in water and kept on ice for 10 min. The cells were pelleted and supernatant fluid collected (periplasmic fraction) and stored at 4°C. Pellets containing the spheroplasts were suspended in 10 mM HEPES buffer, pH 7.4, containing 10 mM EDTA, sonicated for 2 min (30 s bursts) and centrifuged at $1000 \times g$ for 10 min. The resulting supernatant fluid was collected and stored at 4°C (cytosolic fraction).

Protein assays. Protein concentration was determined using the Pierce BCA Protein Assay according to manufacturer's instructions (Pierce, Rockford, IL). Purified CT-A (List Biologicals, Campbell, CA) was used as a positive control on all protein gels, Western blots and ADP-ribosylation assays.

ADP-ribosyltransferase activity assay. Periplasmic and cytosolic fractions were assayed for the presence of ADP-ribosyltransferase activity using an agmatine assay as previously described.[23] Briefly, activity of cholera toxin was measured by the glycohydrolytic cleavage of the ^{14}C-labeled NAD donor substrate into nicotinamide and ADP-ribose, and the subsequent transfer of the ADP-ribose moiety to the acceptor substrate agmatine (1-amino-4-guanidinobutane; Sigma Chemical Co., St. Louis, MO). Assays were performed in 300 μl volumes containing 50 mM KPO_4 buffer, pH 7.5, 5 mM $MgCl_2$, 0.2 mM GTP, 20 mM DTT, 10 mM agmatine, 0.1 mg/ml ovalbumin, and 1 μCi[(U-^{14}C)adenine]NAD (Amersham Corp., Arlington Heights, IL). Reactions were initiated by the addition of equivalent amounts of protein and allowed to proceed for one hour at 37°C. Two 100 μl aliquots from each reaction were applied to Bio-Rad AG1 X-2 anion exchange columns and each column was washed with 5 ml water. Effluent was collected directly into scintillation vials and counted. The positive control contained 2 μg purified CT-A protein.

Limited proteolysis. Purified CT-A and periplasmic fractions containing unmodified recombinant protein or the BL21:R7K protein were subjected to limited tryptic digestion for periods of up to 1 h. Fifty micrograms of each protein sample were digested with 10 μg/ml trypsin in 25 mM HEPES, 1 mM $CaCl_2$, and 0.5 mM EDTA at room temperature for 15, 30, 60 or 90 min. Proteolysis was stopped by the addition of phenylmethylsulfonylfluoride (1 mM final concentration) and aliquots of each sample were electrophoresed and immunoblotted as described above.

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